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# *Telomerase activity in bleomycin-induced epithelial cell apoptosis and lung fibrosis*

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**Abbreviated Title:** Telomerase in lung epithelial apoptosis & Fibrosis

**Abstract**

Epithelial cell injury and apoptosis are recognized as early features in idiopathic pulmonary fibrosis and bleomycin-induced fibrosis in mice. Telomerase is a known apoptosis-alleviating factor. We studied the role of telomerase during bleomycin-induced lung epithelial cell (LEC) apoptosis *in vitro* in a mouse LEC line, and *in vivo* in LECs isolated from bleomycin-treated mice.

We evaluated changes in mTERT mRNA levels and changes in telomerase activity with the TRAPeze detection kit, telomeric length with the TeloTTAGGG Telomere Length Kit, and LEC apoptosis with FACScan and DAPI stain.

Twenty-four hours after bleomycin treatment *in vitro*, there was a significant elevation in mTERT mRNA and a transient 41% increase in telomerase activity. At 72 hours, telomerase activity had fallen to 26% below levels in untreated cells. Reduction of telomerase activity over time, or by direct inhibition, significantly elevated LEC apoptosis. No change in average telomeric length was noted. *In vivo*, telomerase activity of LECs from bleomycin-treated mice increased at 7 and 14 days.

We conclude that telomerase activity may play a protective role from robust bleomycin-induced lung epithelial cell apoptosis. Moreover, stabilizing telomerase activity may decrease epithelial cell apoptosis and the resulting lung fibrosis.

**Keywords:** apoptosis, bleomycin, epithelial cells, fibrosis, lung, telomerase

## Introduction

Bleomycin-induced lung injury in mice is a well-established model for lung fibrosis [1]. Following *in vivo* instillation of bleomycin to the trachea, apoptosis is induced in lung epithelial cells losing their potential to reconstitute a normal alveolar surface [2]. When this occurs, normal lung epithelial cells are replaced with fibroblasts, leading to fibrosis [2-4]. Inhibition of apoptosis could diminish epithelial cell replacement by fibroblasts, and thus reduce lung fibrosis.

Telomere length is maintained by telomerase, a ribonucleoprotein RNA-dependent DNA polymerase complex. Telomerase consists of an RNA template and a catalytic protein, telomerase reverse transcriptase (TERT) [5]. Maintenance of telomerase activity is essential for the survival of proliferating cells [6-8]. Knockout mice lacking the gene for TERT express higher levels of apoptosis and show liver fibrosis, both of which can be repaired by the introduction of telomerase [9].

We have recently confirmed that bleomycin causes apoptosis of lung epithelial cells *in vitro* in a time- and dose-dependent manner [10]. We have also reported the specific induction of epithelial cell apoptosis by myofibroblasts from fibrotic lungs via the Fas/FasL pathway [11]. In the current study, we aimed to evaluate telomerase activity in bleomycin-treated lung epithelial cells (LEC), and the resulting effect on apoptosis in bleomycin-treated MLE cells. We hypothesized that bleomycin injury would result in a reduction of telomerase activity in lung epithelial cells, in contrast to fibroblasts, and thus an increase in LEC apoptosis.

We found that bleomycin causes an initial increase, and then a reduction in telomerase activity, controlled, at least in part, at the mRNA level. When telomerase activity is diminished, significant apoptosis of epithelial cells is initiated. With further disruption of telomerase activity, apoptosis occurs at significantly higher levels.

In order to establish the relevance of our findings to the pathological process of lung fibrosis, we evaluated telomerase activity *in vivo* in an experimental model of murine bleomycin-induced fibrosis. We found an early reduction of telomerase activity in lung

epithelial cells isolated from bleomycin-treated mice. This was followed by a pattern similar to that encountered *in vitro*, with significant elevation of telomerase activity in the surviving cells, followed eventually by a reduction back to baseline levels.

## Materials & Methods

### Cell Line and Culture

Experiments were conducted with the murine type II, SV40-transformed lung epithelial cell line (ATCC, MLE-15). This extensively used cell line [11-13] was maintained in HITES (Ham's F12, insulin, transferin, ß-estradiol, and sodium selenite) medium, supplemented with 2% fetal bovine serum (FBS) (Biological Industries, Beit Haemek, Israel).

### Animals

Male C57B1/6, 11-12-week old mice, each weighing 25-35g (Harlan Sprague – Dawley, Indianapolis, IN), were used. The local committee for animal experiments approved all animal care and experimental protocols.

### Reagents

The reagents used were: propidium iodide (PI) stock solution, 1 mg/ml (Calbiochem, La Jolla, CA) in phosphate-buffered saline (PBS); FITC-conjugated Annexin V (BD Pharmingen, San Diego, CA); bleomycin (ASTA Medica, Germany); tetra(N-methyl-4-pyridyl)-porphyrin chloride (TMPyP4), a G-quadruplex-intercalating porphyrin telomerase inhibitor (Calbiochem, Cambridge, MA); 4,6-diamino-2-phenylindole dihydrochloride (DAPI) stain (Sigma-Aldrich, St Louis, MO); pentobarbitol 6% (Shoresh Pharm, Israel); dispase II (Roche Diagnostics GmbH, Mannhein, Germany); DMEM (Sigma-Aldrich); DNAse (Sigma-Aldrich); 4% paraformaldehyde (Sigma-Aldrich); and saponin buffer (Sigma-Aldrich).

### Exposure of Mouse Lung Epithelial (MLE) Line Cells to Bleomycin

MLE cells suspended in HITES medium were incubated with or without 0.1 unit/ml bleomycin. This bleomycin dose was selected based on previous kinetic studies in our laboratory, because at 24 hours it induces significant, but not overwhelming, apoptosis [10]. After incubation, trypsin (Biological Industries, Beit Haemek, Israel) was added for adherent cell removal, and the mixture was centrifuged (1200 rpm, 10 min). The pellet

was resuspended for further evaluation, and viable cells were counted using trypan blue (Sigma-Aldrich).

**Evaluation of Apoptosis**

DAPI staining was used to assess the percentage of apoptotic cells, as previously described [12, 14]. After elution of the media, MLE cells were washed with PBS (5 min/RT). Cold methanol (2ml) was added to each plate, cells were reincubated (30 min/–20°C) and then rewashed, and sediment was placed on coverslips. DAPI stain (100µl of 1µg/ml) was added to the coverslips, which were then incubated in the dark (15 min/ RT), washed twice with PBS, and dried. The coverslips were placed on slides that had been prepared in advance with 20µl of mounting solution. Slides were evaluated with a fluorescent microscope (Axiovert 200, Carl Zeiss AG, Oberkochen, Germany), and data was analyzed (Image-Pro Plus 4.1, MediaCybernetics, Silver Spring, MD). At least one hundred cells were randomly selected and counted from each slide, and the average percentage of apoptotic cells from all slides in each group was reported.

Flow cytometry analysis with FACScan (Becton Dickinson, Mountain View, CA) was used in one of two ways as a second method to evaluate apoptosis:

1. Cells ($10^6$/ml) were incubated (15 min/RT) with 5µl/ml of FITC-conjugated Annexin V, collected, and pelleted. Propidium iodide (1µg/ml) was added to the cells (5 min/RT). Flow cytometry analysis (FL1/FITC-Annexin V plotted against FL2/PI positive cells) was performed with FACScan, and data was analyzed with CellQuest-ProTM software (Becton Dickinson). PI-positive cells were excluded from the presented data.

2. Due to the autofluorescence of TMPyP4, Annexin V measurement was not possible when TMPyP4 was used. In this situation, cells ($10^6$/ml) were evaluated for size and granularity (FSC and SSC) using FACScan. The population of apoptotic cells was characterized by their smaller size, implying apoptotic shrinkage, as well as their higher granularity, representing apoptotic cytoplasmic condensation, as previously reported [15, 16].

**RNA Isolation**

Total cellular RNA was isolated from cell pellets using TRI Reagent (Sigma-Aldrich) supplemented by 1μg/ml of glycogen, according to the protocol supplied by the manufacturer. To assess RNA integrity and verify the absence of a high molecular weight band representing contamination with genomic DNA, an aliquot of each sample was analyzed by 1% agarose gel electrophoresis. RNA was also analyzed by spectrophotometer for assessment of integrity, protein contamination (A260:A280 >1.8), and quantification.

**Semiquantitative RT-PCR of mTERT**

RNA was reverse-transcribed to cDNA using an avian myeloblastosis virus RT-based protocol with random primers, as well as poly dT (Reverse Transcription System, Promega, Madison, WI). One μg of each sample was uniformly used for reverse transcription. Taq DNA polymerase (0.25ml) (Roche Diagnostics GmbH) was added to each tube following hot start incubation (5 min, 95°C). This was followed by 40 cycles for mTERT and 28 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) denaturation (15sec, 96°C), annealing (20sec at 65°C), extension (1 min, 72°C), and a final extension step (5 min). Two μl of forward and reverse primer sequences (Sigma-Aldrich) were used as follows:

forward mTERT: 5'-GGGAGATGGCCAAGAGCGTCTAAA-3'

reverse mTERT: 5'-CGGTGGGCTGGTGTTCAAGG-3'

forward GAPDH: 5'-GTTGCCATCAATGACCCCTTC-3'

reverse GAPDH: 5'-CATGTGGGCCATGAGGTCCAC-3'

The number of cycles was predetermined to be the greatest number in which amplification was within the linear range. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium-bromide (Sigma-Aldrich). The intensity of the fluorescent signal emitted by the PCR products was determined by

densitometry using a tabletop scanner (Multi-Analyst$^{TM}$ / PC Version 1.1, Bio-Rad Laboratories, Hercules, CA). Data was analyzed with the Fluor-S-MultiImager (Bio-Rad). Each cDNA was amplified in triplicate, corrected to the level of GAPDH mRNA, and the median value used. Amplification was repeated with a smaller quantity of substrate if the densitometer signal was beyond the predetermined linear range.

**Detection of Telomerase Activity**

Telomerase activity was detected using the TRAPeze Telomerase Detection Kit (Intergen, Purchase, NY), a PCR-based Telomeric Repeat Amplification Protocol (TRAP) method, as previously described [17]. Briefly, cell extracts were resuspended in CHAPS lysis buffer and combined with the reaction mix in RNAse-free PCR tubes, gaining telomerase extension. Positive and negative controls were examined accordingly. Protein levels were evaluated using the Bradford method, and equal amounts of protein were evaluated. PCR amplification was then performed. Loading dye was added to each reaction tube, and the mixture was loaded onto a 12.5% non-denaturizing SDS PAGE gel. Following electrophoresis, the gel was stained with ethidium bromide, and detection was determined by densitometry using a tabletop scanner (Bio-Rad). The total and relative quantities of telomerase products for each sample was calculated using the Fluor-S-MultiImager.

**Measurement of Terminal Restriction Fragment (Telomeric) Length**

Telomeric length was determined as previously described [17, 18] using the TeloTTAGGG Telomere Length Assay Kit (Roche Diagnostics GmbH). Briefly, genomic DNA was isolated and digested with *Hinf 1 / Rsa*, DNA fragments were separated by electrophoresis on agarose gel, and Southern Blot transfer was performed. The membrane was hybridized with a telomere-specific digoxigenin (DIG)-labeled probe, incubated with anti-DIG alkaline phosphatase, and assessed with chemiluminescence detection (Kodak X-OMAT 2000 Processor, Kodak, Rochester, NY). Average telomeric length was determined using Telometric 1.2 (Fox Chase Cancer Center, Philadelphia, PA).

**Telomerase Inhibition**

Telomerase activity was inhibited in MLE cells using 1.5 mM of TMPyP4, and inhibition was confirmed using the TRAPeze Telomerase Detection Kit.

**Intratracheal Instillation of Animals**

Intratracheal instillation of bleomycin or saline to mice was done as we have previously described [1, 19]. Briefly, mice were anesthetized intraperitoneally. The trachea was exposed and a metal cannula was carefully inserted into the trachea. Bleomycin (0.08mg) in 0.1 ml of 0.9% saline, or saline alone was injected.

**Lung Epithelial Cell Isolation for *ex vivo* Culture**

LECs were isolated as we have previously reported [11]. Briefly, animals were sacrificed by aortic transection while under pentobarbitol 6% anesthesia. The lungs were filled with 3 ml dispase II, and allowed to collapse. Low melt agarose (0.5 ml) was then infused, and lungs were covered with ice for 2 minutes. The lungs were excised from the animals and incubated in 1ml dispase II in a 12 ml polypropylene culture tube (45 min/RT). The lungs were transferred to 7 ml DMEM with 0.01% type II DNAse in a 60 mm petri dish. Digested tissue was gently swirled for 5-10 min. The resulting suspension was filtered through 100 μm and 40 μm Falcon strainers, and then through a 22 μm nylon mesh, centrifuged (900RPM/12 min/RT), and resuspended in culture medium. The cells were placed on culture plates previously coated with mouse anti-CD45 and mouse anti-CD16/32 monoclonal antibodies (IQ Products, Groningen, The Netherlands). Cells were incubated (2 hr/37°C), transferred to new uncoated culture plate cells (45 min/37°C), and collected by centrifugation.

**Evaluation of Isolated Lung Epithelial Cells for Purity by Flow Cytometry**

In order to assess the purity of isolated epithelial cells, both extracellular and intracellular markers were evaluated:

1. Extracellular staining – LECs were fixed with 4% paraformaldehyde (10 min/RT) and then washed with PBS. LECs were then incubated (30 min/RT) with 10 μl/100 μl of one of four antibodies: of FITC-conjugated lymphocyte

marker (anti-CD3, IQp Products); leukocyte marker (anti-CD31, BD Pharmingen, San Diego, CA); macrophage marker (F4/80, Serotec, Raleigh, NC); type I epithelial cell marker (AQ5, non-FITC conjugated, with addition of secondary antibody FITC-conjugated anti-goat, Jackson Immunoresearch Laboratories, West Grove, PA). The cells were washed twice in saponin buffer and analyzed by flow cytometry.

2. Intracellular Staining – Cells were washed with 0.1% saponin buffer and then incubated (30 min/RT) with 1μl/100μl of one of the following antibodies: type II epithelial markers (anti-SFB or anti-SFC, Chemicon, Temecula, CA); myofibroblast marker (anti-αSMA FITC conjugate, Chemicon). For SFC and SFB staining, 1μg/100μl of FITC conjugated anti-rabbit antibodies (Jackson Immunoresearch Laboratories) was added and cells were reincubated (30 min/RT). The cells were then washed twice in saponin buffer and analyzed by flow cytometry.

Following this two-step evaluation, about 90% of the isolated cells were found to be type I or II lung epithelial cells.

**Statistical Analysis**

Analysis of variance was performed with the Kruskal-Wallis test for nonparametric data. When Kruskal-Wallis tests of comparability were statistically significant, Mann-Whitney comparisons with Holms sequential Bonferroni corrected p-values were performed. In order to determine whether relative telomerase activity was consistently greater, or lesser than baseline, the data was dichotomized. One sample chi-square test was performed, comparing the observed distribution to an expected random distribution. $p<0.05$ was considered significant. Statistical analysis was performed with GraphPad Prism software for Windows (GraphPad Software, San Diego, CA).

## Results

### Bleomycin Modifies Telomerase Activity *in vitro*

In order to study the effects of bleomycin on telomerase activity of lung epithelial cells, MLE cells were exposed to bleomycin for 24, 48, or 72 hours. Cells were collected and resuspended. Viable cells were counted using trypan blue in order to exclude necrotic cells. A similar number of viable cells were taken for evaluation of telomerase activity from each group of bleomycin-treated and untreated cells. Telomerase activity increased in bleomycin-treated cells, compared to untreated control cells, by a mean of 41% and 12% at 24 and 48 hours respectively. The mean activity then decreased to 26% below control levels at 72 hours (Figure 1). At 96 hours, bleomycin caused excessive cell death, precluding measurement in four of six experiments. However, in two successful experiments the mean reduction of telomerase activity at 96 hours in bleomycin-treated compared to control cells was 43% (data not shown). Figure 1 shows the level of telomerase activity in bleomycin-treated compared to untreated control cells at each time point. A regression analysis of the percent of change versus time of bleomycin exposure yielded the following linear equation: fold ratio = 1.771 – 0.307 x exposure days ($r^2$=0.83, p=0.023). The level of telomerase activity in bleomycin-treated cells compared to untreated control cells was significantly higher at 24 hours (p=0.014), and lower at 72 hours (p=0.014).

### Bleomycin Increases Expression of mTERT mRNA

The transcriptional regulation of the catalytic component of telomerase (mTERT) is recognized as one of the major rate-limiting steps in the induction of telomerase activity [5]. In order to assess the regulation level of telomerase activity in bleomycin-treated MLE cells, mTERT mRNA levels were measured. Cells were exposed to bleomycin for 24, 48, or 72 hours. mTERT mRNA in bleomycin-treated MLE cells was measured by semi-quantitative RT-PCR, and compared to the median level of untreated control cells at each time point. Bleomycin induced a substantial increment of mTERT mRNA at 24 hours. There was some elevation at 48 hours, though considerably less significant. The

level of mTERT mRNA in bleomycin-treated MLE cells returned nearly to control levels at 72 hours (Figure 2).

**Bleomycin Does Not Change Average Telomeric Length**

Telomerase inhibition can induce apoptosis in several ways, however telomere shortening is generally accepted to be the primary mechanism [7]. In order to assess bleomycin-induced changes in telomeric length, the average terminal restriction fragment length was measured using the TeloTTAGGG Telomere Length Assay Kit (Roche Diagnostics, GmbH). In three independent experiments, there was no difference between average telomeric length in MLE cells exposed to bleomycin compared to controls at 24 or 72 hours. The results shown in Figure 3 are representative of four experiments.

**Elevation in Telomerase Activity Delays Robust Apoptosis**

We performed kinetic evaluations of telomerase activity levels and apoptosis rates in bleomycin-treated MLE cells at 6-48 hours. Telomerase activity was assessed with the TRAPeze Telomerase Detection Kit, and the percentage of apoptotic cells was estimated using double staining with Annexin V and PI. A representative experiment is shown in Figure 4. There was an initial elevation of relative telomerase activity in bleomycin-treated cells, followed by a decrease in activity that was associated with a significant increase in apoptosis.

**Inhibition of Telomerase Activity by TMPyP4 Increases Epithelial Cell Apoptosis**

TMPyP4 is a known G-quadriplex inhibitor of telomerase activity [20]. TMPyP4 exposure alone caused apoptosis in MLE cells at rates similar to bleomycin exposure (data not shown). In order to establish the relationship between changes in telomerase activity and apoptosis, bleomycin-treated MLE cells were exposed to TMPyP4 for 24 hours. TMPyP4 was confirmed to inhibit the bleomycin-induced elevation in telomerase activity using the TRAPeze Telomerase Detection Kit (data not shown). Apoptosis, evaluated using the DAPI stain, was higher in bleomycin-treated MLE than untreated control cells, and increased appreciably in bleomycin-treated MLE cells that were exposed to the telomerase inhibitor TMPyP4 (Fig. 5a). For quantification, the proportion

of apoptotic cells was calculated in 200 random cells on every DAPI-stained slide, in four independent experiments. The mean percent of MLE apoptotic cells was 81.3% in bleomycin and TMPyP4-treated cells compared to 43.4% in cells treated with bleomycin alone ($p<0.05$, Fig. 5b).

Apoptosis was further evaluated using flow cytometry analysis of size and granularity as previously described [15, 21]. Cells exposed to both bleomycin and telomerase inhibition had a significantly higher proportion of apoptotic cells when compared to treatment with bleomycin alone (Figure 5d). A representative dot plot is presented in Figure 5c.

**Bleomycin Modifies Telomerase Activity in Lung Epithelial Cells *in vivo***

Epithelial cells were isolated from mouse lungs 1, 3, 7, 14, and 21 days following intratracheal instillation of bleomycin or saline. The development of fibrosis was confirmed in bleomycin-treated mice as previously reported [1]. Changes in telomerase activity of LEC isolated from bleomycin-treated compared to saline-treated mice are represented in Figure 6. Telomerase activity decreased at day 3 by a mean of 59% ($p=0.046$). At day 7 there was a mean elevation of 36% in telomerase activity in surviving LEC ($p=0.046$), with a further increase at day 14 by a mean 68% ($p=0.014$). No significant change in telomerase activity in bleomycin-treated compared to control mice was found at day 21, when the fibrotic process subsided (Fig 6).

## Discussion

In the current work we studied the role of telomerase during bleomycin-induced apoptosis in lung epithelial cells *in vitro*, and in lung epithelial cells (LEC) isolated from mice with bleomycin-induced lung fibrosis *in vivo*.

We found *in vitro* that bleomycin causes an initial elevation of telomerase activity, followed by a reduction, in murine lung epithelial (MLE) cells. Initial bleomycin-induced apoptosis, has occurred at 12 hours, as telomerase activity increases. Subsequent reduction of telomerase activity over time results in substantially increased apoptosis (Fig. 1).

In order to establish the relevance of our findings to the evolution of lung fibrosis, we evaluated telomerase activity *in vivo* in LECs isolated from bleomycin-treated mice. At day 7, and even more so at day 14, LECs isolated from bleomycin-treated mice showed a significant elevation of telomerase activity compared to LECs from saline-treated control mice. Increased telomerase activity is possibly a mechanism that enables these cells to outlive the attack of neighboring inflammatory and other effector cells. When the fibrotic process subsided, at day 21, telomerase activity dropped down to the levels found in control mice.

Mounting evidence indicates that telomerase activity protects cells from apoptosis via multiple mechanisms [7, 8, 22, 23]. In the current study, we show the anti-apoptotic effects of telomerase in murine lung epithelial cells, where a transient, bleomycin-induced elevation of telomerase activity contributed to a significant delay in bleomycin-induced apoptosis (Fig. 4). Klapper, *et al.* reported a similar transient elevation in telomerase activity induced in HL-60 cells by etoposide, another DNA damaging agent [24].

Early apoptosis is mediated by the process of DNA damage [10, 25], and telomerase activity does not protect from apoptosis at these early time points. Telomerase activity increases only after apoptosis has initiated (Fig. 4). It is possible that apoptosis is the trigger for the induction of telomerase as a defense mechanism from bleomycin-induced

robust apoptosis. Therefore the initial increase in bleomycin-induced telomerase activity, possibly mediated by apoptosis, may protect MLE cells from further apoptosis. Indeed, when the bleomycin-induced increase in telomerase activity diminishes over time (Fig. 1), the level of bleomycin-induced apoptosis increases significantly. The fact that robust apoptosis is induced by specific inhibition of telomerase with TMPyP4 (Fig.5), strengthens our hypothesis that this response is not merely correlative.

We have previously shown that bleomycin causes significant DNA damage in MLE cells [10]. Masutomi, *et al*. showed that suppression of human TERT (hTERT) abrogates the cellular repair response to DNA double-strand breaks [26]. It is therefore reasonable to assume that telomerase activity is elevated as part of the cellular reaction to that damage. Our work is further supported by results presented by Rubio, *et al.*, who showed that telomerase expression and elevation of its activity increased the resistance of cell lines to bleomycin [27]. Taken together, these results suggest that resistance to bleomycin injury could be related, at least partly, to the inhibition of apoptosis induced by the telomerase complex. Our data analysis, using propidium iodide staining followed by flow cytometry, excludes necrotic and dying cells, as opposed to apoptotic cells. This process argues against the possibility that changes in telomerase activity are merely a toxic effect of bleomycin. Elevations in telomerase activity and mRNA support our concept that this reaction is part of an active phenomenon.

Telomere shortening is the main mechanism for apoptosis induction following telomerase inhibition [7]. Massive telomere loss has been shown to occur early in DNA-damage-induced apoptosis [28]. However, accumulating evidence suggests that telomerase also plays an important role in protecting karyotypic stability, independent of its part in maintaining telomeric length [22]. Rubio, *et al.* reported that resistance to injury was related to telomerase activity only in cell lines with short telomeres, suggesting that telomere shortening is the cause of apoptosis [27]. In contrast, we found no change in the average length of telomeres in bleomycin-treated MLE cells up to 72 hours, a time by which significant apoptosis has typically occurred. This finding agrees with the accepted convention that telomere shortening is a slow-acting mechanism for apoptosis induction [5]. It is possible that a limited and more specific telomeric loss (i.e. in a specific

chromosome) is responsible for the induction of robust lung epithelial cell apoptosis. However, it is clear that general massive telomere loss is probably not primarily responsible for apoptosis induction in these cells. Two rapid mechanisms of telomerase activity been described, and could account for the protection from apoptosis without notable change in telomeric length. These are telomere capping [7, 29], and regulation of gene expression by telomerase [23]. Induction of telomerase activity has been shown to significantly influence the expression of several oncogenes [30], and the absence of telomerase could activate genes involved in apoptosis [7].

The transcriptional regulation of the catalytic component of telomerase expression is recognized as one of the major rate-limiting steps in the induction of telomerase activity [5]. We found a rapid, substantial elevation in mTERT mRNA as early as 24 hours after treatment with bleomycin. The bleomycin-induced elevation in telomerase activity is therefore regulated at least in part by this transcription.

Measurements of telomere elongation were the method used to assess telomerase activity. These measurements revealed a modest increase in telomerase activity at 24 hours (Fig.1), versus a substantial elevation of mTERT mRNA (Fig. 2). The lack of correlation between these measurements supports our concept that telomerase protected LECs from apoptosis in a manner unrelated to telomere elongation and telomeric loss.

Increasing evidence suggests that lung epithelial cell apoptosis is an important contributor to bleomycin-induced lung fibrosis [2, 31]. One of the main theories for the pathophysiology of idiopathic pulmonary fibrosis (IPF) is that injury to the pulmonary parenchyma causes epithelial cell damage and apoptosis [32]. Evidence of type II pneumocyte apoptosis in normal alveoli of IPF patients has been reported [33], and loss of alveolar epithelium, with failure of reepithelization, was suggested to stimulate persistence and progression of fibrosis [32].

We have recently shown that myofibroblasts, which are known to accumulate abundantly in the lungs of mice with bleomycin-induced fibrosis [3, 34], are not only collagen producers, but function also as effector cells [11]. We demonstrated that they induce

apoptosis of lung epithelial cells *in vivo*, a possible mechanism for the prevention of lung tissue remodeling [11]. Apoptosis thus plays a role in the induction of fibrosis.

In the current work, we found that telomerase activity may have a role in bleomycin-induced lung fibrosis *in vivo*. Shortly after bleomycin injury, telomerase activity in LECs decreases. However, at days 7 and 14, telomerase activity increases, enabling epithelial cell regeneration, while epithelial cells without elevated telomerase activity may have already undergone apoptosis. Since elevated telomerase activity has been repeatedly shown to assist cell survival [7, 8, 22, 23], it is to be expected that during intensive fibrosis (on days 7 and 14), surviving epithelial cells have levels of telomerase activity that are higher than baseline (Fig. 6). We suggest that this elevation in telomerase activity is a cellular defense mechanism, against active myofibroblast-induced lung epithelial cell apoptosis.

We have shown in the past that the fibrotic process in bleomycin-treated C57Bl/6J mice subsides at day 21 [1]. It is therefore not surprising that, at this time point, when the lung regeneration process has advanced and elevation of telomerase activity confers no survival advantage, it returns to normal levels in the remaining epithelial cells.

The impact of bleomycin-induced injury on telomerase activity is different in lung fibroblasts and epithelial cells. Nozaki, *et al*. noted a constant elevation in telomerase activity in fibroblasts following bleomycin instillation with extended life span [4]. In contrast, we show that epithelial cells have only a transient elevation of telomerase activity, followed by a significant reduction in it, resulting in robust apoptosis.

The most important question arising from our work is the relationship between changes in telomerase activity and human lung fibrosis. Mutations in the telomerase complex are linked to dyskeratosis congenita, a rare, inherited syndrome of bone marrow failure [35]. Pulmonary fibrosis is a recognized manifestation of this syndrome [36]. Furthermore, strong support for the relationship between an altered telomerase complex and IPF has been very recently published, demonstrating that mutations in the genes encoding telomerase can appear in familial idiopathic pulmonary fibrosis [37]. Our work on epithelial cells, together with other research done on fibroblasts [3, 8], suggests the a

possibility of a relationship between telomerase mutations and lung fibrosis. Thus changes in telomerase activity could alter the differential reaction of pulmonary epithelial cells and fibroblasts to lung injury.

Our results demonstrate that changes in telomerase activity, regulated at least in part by transcription, play a role in lung epithelial cell apoptosis by a mechanism that is independent of telomere loss. The elevation of telomerase activity in LECs *in vivo,* may represent an active defense process in surviving lung epithelial cells against neighboring myofibroblasts that may cause apoptosis of epithelial cells as part of the fibrotic process. Those epithelial cells that activate a defense mechanism, including, but probably not limited to elevation of telomerase activity, could escape apoptosis, survive the injury, and help reduce the extent of fibrosis.

We therefore present a novel possibility for regulation of DNA damage-induced lung injury through modifying telomerase activity. Understanding the role of telomerase in apoptosis of epithelial cells is an important step in the evaluation of lung injury, and possibly of fibrosis related to DNA damage. Therefore, stabilizing telomerase activity in lung epithelial cells may decrease epithelial cell apoptosis and eventual lung fibrosis. This hypothesis is strongly supported by the recent reports that mutations in genes encoding telomerase have been found in familial IPF [37].

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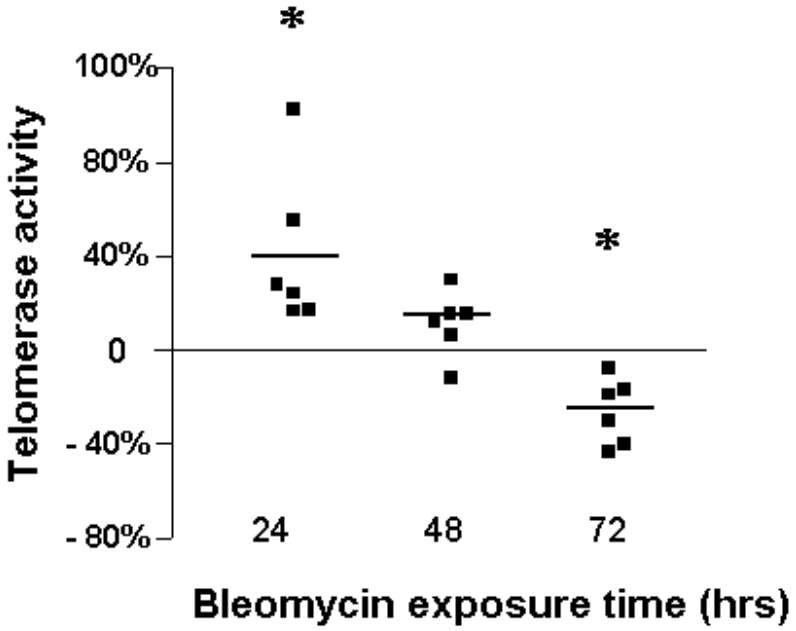
*
100%
80%
40%
0
- 40%
- 80%
Telomerase activity
24
48
72
*
Bleomycin exposure time (hrs)

Fig. 1 -

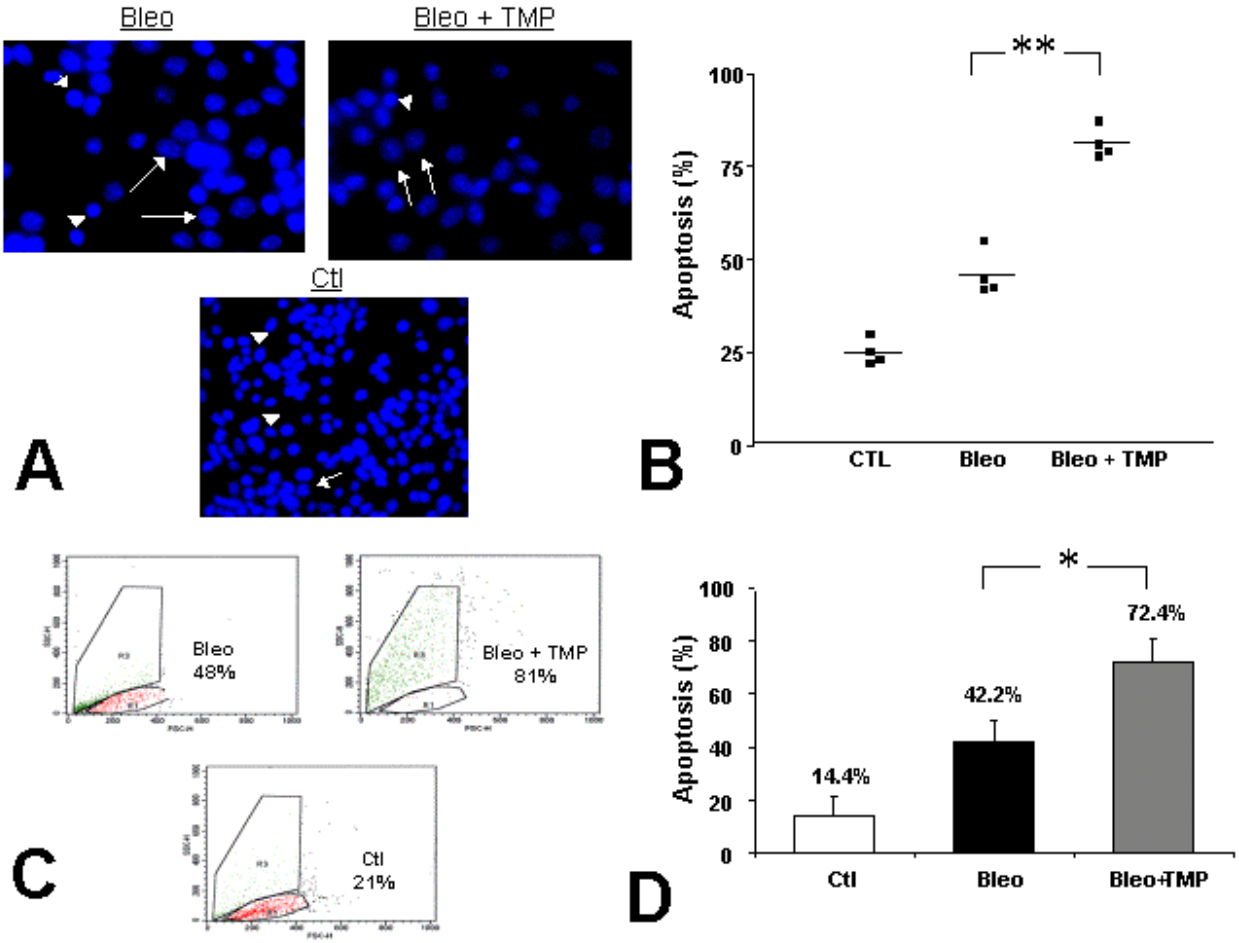
Bleo
Bleo + TMP
Ctl
A
B
**
Apoptosis (%)
100
75
50
25
0
CTL
Bleo
Bleo + TMP
C
Bleo
48%
Bleo + TMP
81%
Ctl
21%
D
*
Apoptosis (%)
100
80
60
40
20
0
14.4%
42.2%
72.4%
Ctl
Bleo
Bleo+TMP

Fig. 5 -

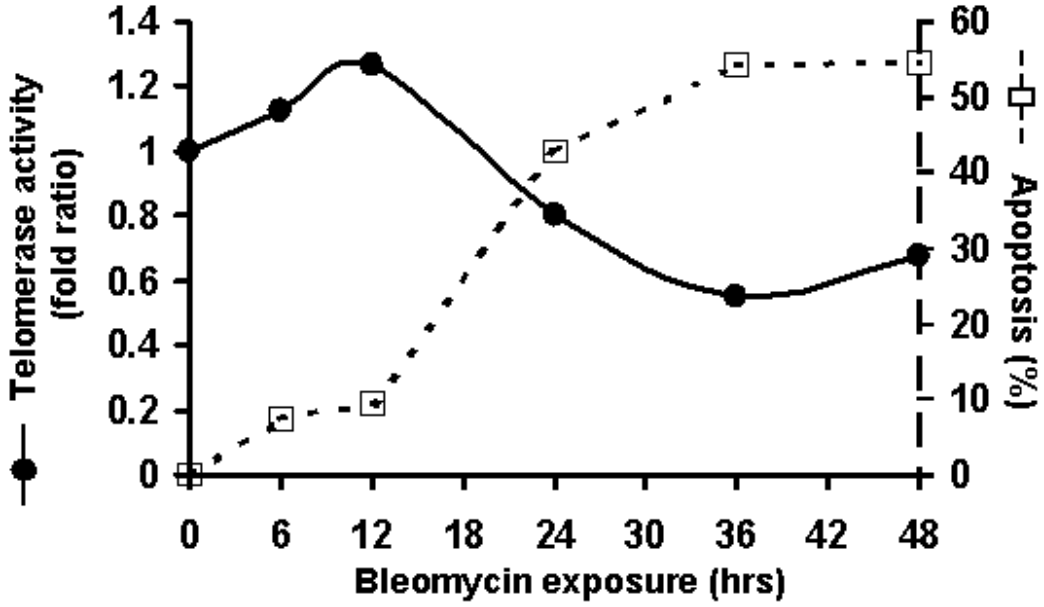
Telomerase activity
(fold ratio)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Apoptosis (%)
60
50
40
30
20
10
0
0
6
12
18
24
30
36
42
48
Bleomycin exposure (hrs)

Fig. 4 -

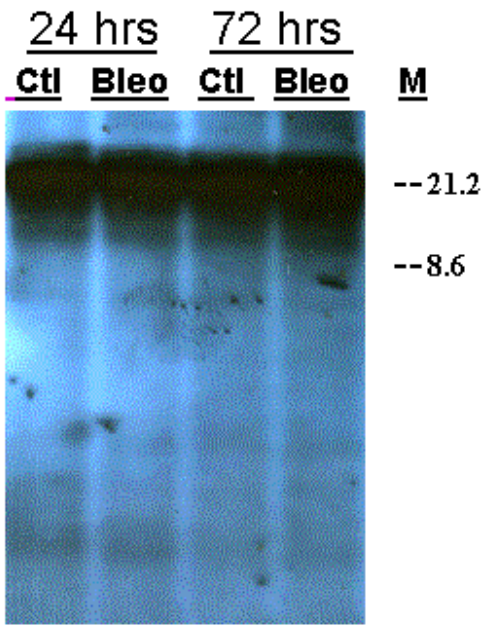


Fig. 3 -

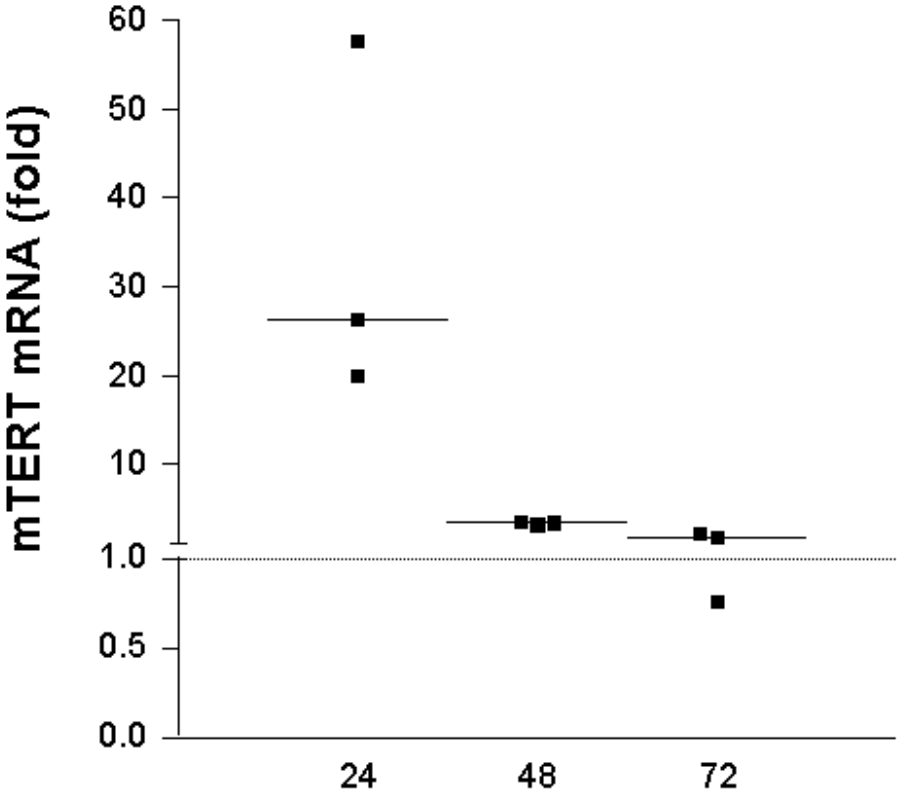
60
50
40
30
20
10
1.0
0.5
0.0
mTERT mRNA (fold)
24
48
72
Bleomycin exposure time (hrs)

Fig. 2 -

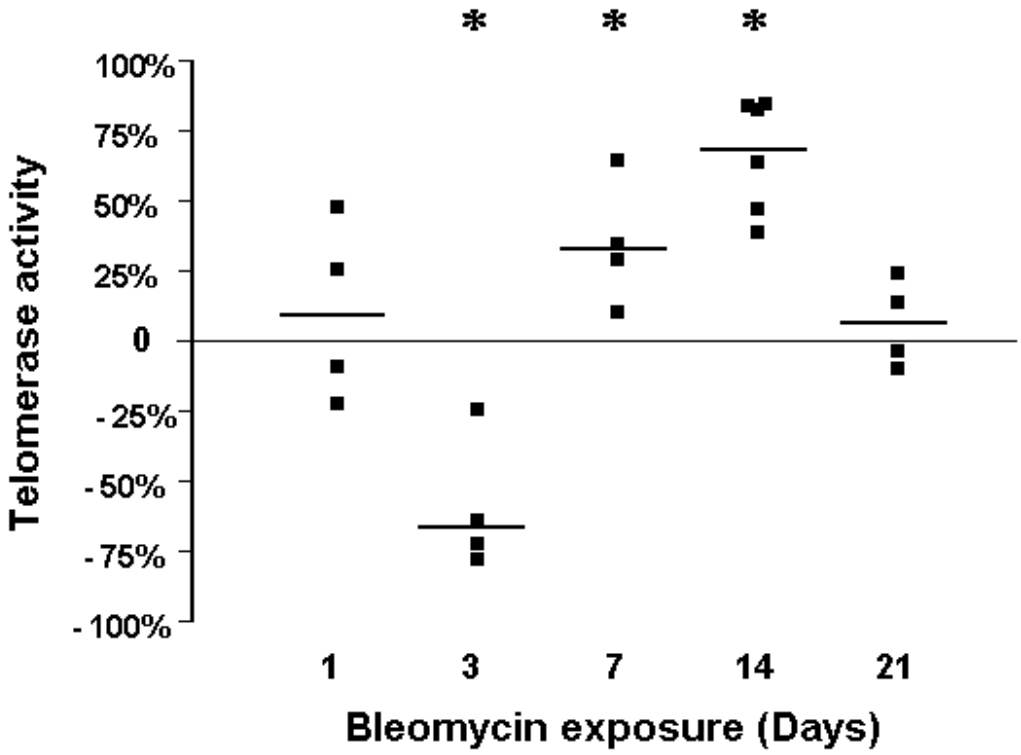
*
*
*
100%
75%
50%
25%
0
-25%
-50%
-75%
-100%
Telomerase activity
1
3
7
14
21
Bleomycin exposure (Days)

Fig. 6 -

## Figure Legends

**Figure 1 – Bleomycin modifies telomerase activity** – Telomerase activity was assessed in bleomycin-treated and untreated MLE cells using the TRAPeze Telomerase Detection Kit. The percent of change in telomerase activity in bleomycin-treated cells, compared to untreated control cells at 24, 48, and 72 hours is presented. Each point represents an independent comparison between bleomycin-treated and control cells

* $p<0.05$.

**Figure 2 – Bleomycin increases expression of mTERT mRNA –** The level of mTERT mRNA was assessed in MLE cells using semi quantitative RT-PCR. The ratio between the level of mTERT mRNA in bleomycin-treated cells and the median level of mTERT mRNA in untreated control cells, at each time point, both adjusted to GAPDH, is presented. Each point represents an independent experiment.

**Figure 3 – Bleomycin does not change telomeric length –** A representative experiment showing the results of average telomeric length measurements at 24 and 72 hours. No difference was seen between the calculated average telomeric length in bleomycin-treated MLE cells **(**Bleo**)** and untreated control cells **(**Ctl**). M** – size marker.

**Figure 4 – Elevation in Telomerase activity Delays Robust Apoptosis –** Telomerase activity at 6-48 hours, assessed with the TRAPeze Telomerase Detection Kit, is expressed as the ratio of bleomycin-treated to untreated control MLE cells (—●—), graded on the left axis. The percent of apoptosis in bleomycin-treated cells (- - □ - -), as evaluated with Annexin V / PI staining, is graded on the right axis.

**Figure 5 – Inhibition of telomerase activity increases bleomycin-induced apoptosis –** Using DAPI stain for the identification of apoptotic cells, panel **A** presents the increased percent of apoptotic cells in bleomycin- and TMPyP4-treated MLE cells (Bleo+TMP) at 24 hours, compared to cells treated solely with bleomycin (Bleo). Minimal apoptosis is shown in untreated control cells (Ctl). The speckled cells are apoptotic (arrows), while the smooth cells are nonapoptotic (arrowheads). Panel **B** shows the percent of apoptotic MLE cells at 24 hours in four independent experiments, each represented by a dot. Mean values are presented by solid lines. Panel **C** shows a representative FACS analysis of apoptosis at 24 hours. Apoptotic cells were identified by size and granularity. Panel **D** shows a diagram of mean±SD values representing the percent of apoptotic MLE cells in seven independent experiments.

* $p<0.05$, ** $p<0.01$

R1 – Region of nonapoptotic cells  R2 – Region of apoptotic cells

**Figure 6 – Bleomycin Modifies Telomerase Activity in Lung Epithelial Cells *in vivo* –** Telomerase activity was assessed in lung epithelial cells (LEC) isolated from bleomycin- and saline-treated (control) mice using the TRAPeze Telomerase Detection Kit. The percent of change in telomerase activity of LEC isolated from bleomycin- in relation to saline-treated control mice at 1, 3, 7, 14, and 21 days post intratracheal instillation is presented. Each point represents an independent value.

* $p<0.05$.